

ORIGINAL ARTICLE

Detection of Mycobacteria in Crohn's Disease by a Broad Spectrum Polymerase Chain Reaction

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Background: The role of mycobacterial infection, particularly related to *Mycobacterium avium* subsp *paratuberculosis* (*Map*), in Crohn's disease has long been debated. We developed primer pairs capable of detecting a broad spectrum of mycobacterium and employed them to investigate surgical specimens from patients with Crohn's disease.

Methods: Pan mycobacterium primers of the 65-kDa heat shock protein gene (*Hsp65*) were used in a polymerase chain reaction (PCR) to examine 12 surgically-resected, formalin-fixed, paraffin-embedded specimens from 11 patients with Crohn's disease. The DNA sequences of amplicons were aligned with those in GenBank.

Results: Mycobacterial DNA was found in specimens from three of 11 patients. *M. mucogenicum* was identified in a specimen from one patient and *M. tuberculosis* in two, but *Map* was not identified in any.

Conclusion: *Hsp65*-based PCR can be employed to search for occult mycobacterial infection of the gastrointestinal tract in patients with a diagnosis or suspicion of Crohn's disease. This approach may have a therapeutic implication. [*J Formos Med Assoc* 2006;105(4):290–298]

Key Words: Crohn's disease, heat shock protein gene, *Mycobacterium mucogenicum*, *Mycobacterium tuberculosis*, polymerase chain reaction

Crohn's disease was initially reported as chronic interstitial enteritis by Dalziel in 1913,¹ and then described as regional ileitis by Crohn in 1952.² It is an idiopathic inflammatory bowel disease characterized by noncaseating granulomas with transmural inflammation. However, the resemblance of Crohn's disease to paratuberculosis of ruminants (Johne's disease)^{3,4} implies that *Mycobacterium avium* subsp *paratuberculosis* (*Map*) may cause Crohn's disease in humans. This hypothesis has prompted many investigators to determine if *Map* can be detected in Crohn's disease, particularly after the emergence of polymerase chain reaction (PCR)-based techniques. So far, at least

32 PCR-based studies of Crohn's disease have been reported. Among these, 18 studies have found *Map* DNA,^{5–12} whereas 14 have reported negative results.^{13–19} This discrepancy further adds to the complexity of an already confused pathogenesis of Crohn's disease.

Several possible explanations have been provided for the possible false-negative findings. First, sampling paucimicrobial tissue might yield a DNA extract that contains insufficient template for detection by PCR assay. Second, suboptimal procedures, such as inadequate disruption of bacterial walls and the presence of inhibitors for enzyme reactions, might result in detection failure.

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Third, some PCR products may contain too little DNA to be visualized unless sensitive methods such as hybridization with isotope-labeled probes or amplification by nested PCR are employed. It has been noted, however, that most of the reported PCR assays targeting the repetitive IS900 insertion element in the *Map* genome^{20,21} were very sensitive, particularly when nested PCR was employed. Therefore, the negative or low yield of positive samples probably implies that no (or an insignificant percentage of) patients with Crohn's disease were infected with *Map*.

We speculated that the geographic variation of mycobacteria may provide an additional explanation for this discrepancy. There is no evidence to suggest that *Map* is the only species of mycobacterium that can cause Crohn's disease. In addition, Johne's disease is, in fact, morphologically similar to *M. avium* intracellular infection rather than to Crohn's disease.²² To test this hypothesis, we first investigated whether the nucleotide sequence

polymorphism of the mycobacterial cell wall 65-kDa heat shock protein gene (*Hsp65*) can distinguish a broad spectrum of mycobacterium, and then applied this technique to determine if mycobacteria other than *Map* could be detected in the surgical specimens of patients with Crohn's disease.

Methods

All patients with a diagnosis of Crohn's disease between 1992 and 2003 were identified from the files of the Department of Pathology, Mackay Memorial Hospital, Taipei. This study was conducted according to the guidelines of the hospital's institutional review board. The final study group comprised 12 specimens from 11 patients (Table 1). Each had typical features of Crohn's disease, including clinical presentation, imaging profiles, and histopathologic features. The histo-

Table 1. Summary of patients diagnosed with Crohn's disease

Case	Presentation, treatment and outcome
1	35-year-old female with right lower quadrant pain, a palpable mass and leukocytosis; treated by segmental resection of the cecum and terminal ileum; postoperative intestinal obstruction managed by exploratory laparotomy and lysis of adhesion
2	50-year-old male with ileus and a plain abdominal film showed small bowel dilation with gas retention
3	35-year-old female with intermittent right lower quadrant pain; computed tomography showed an appendiceal abscess with local extension into the right psoas-iliac muscles; treated by segmental resection
4	49-year-old female with chronic diarrhea for 2 years; treated by segmental resection of the small intestine and oral prednisolone; postoperative complications included enterocutaneous fistula, occasional diarrhea, malnutrition, and osteoporosis
5	41-year-old male with a 1-year history of chronic diarrhea, tarry stool and body weight loss; fistula formation in the resected small bowel
6	27-year-old male with a 1-month history of left lower quadrant pain and change in bowel habit; roentgenography showed paralytic ileus with segmental narrowing at the junction between the ileum and jejunum; treated by resection of the ileum
7	64-year-old male with abdominal discomfort initially treated by segmental resection of the cecum and ascending colon; persistent enterocolitis and complications (adhesions, fistulae) treated by numerous abdominal surgeries; diagnosis of Crohn's disease made after a right hemicolectomy during his third hospitalization; prednisolone prescribed intermittently
8	40-year-old male with hollow organ perforation; treated by sigmoid colectomy and ileal resection; died of pneumonia and sepsis
9	49-year-old male with intermittent abdominal pain; treated by partial colectomy, resection of the ileum and oral prednisolone
10	35-year-old male with a 4-year history of diarrhea; treated by right hemicolectomy; symptoms recurred 5 years later and treated by ileoileal bypass
11	22-year-old male with abdominal pain, vomiting and tarry stool; ileal resection for a perforation at the distal ileum; postoperative complications included functional bowel disturbance and constipation

pathologic features included evidence of transmural chronic inflammation with multiple lymphoid aggregates and germinal centers, submucosal fibrosis, lymphatic dilatation, muscular hypertrophy, and submucosal neuronal hyperplasia. Some specimens also showed ulceration, microabscess along the fistular tract, and small granulomas in the mesenteric lymph nodes. Acid-fast stain showed no stainable bacilli in all paraffin sections, and all four specimens that were originally cultured were negative for mycobacteria.

DNA isolation

Both the surgical margin with normal histopathology and the areas exhibiting granulomatous inflammation in hematoxylin and eosin-stained sections of each specimen were circled with a marking pen for DNA extraction. DNA was extracted from formalin-fixed tissue according to previously described procedures.²³ In brief, representative paraffin blocks were cut at 8 µm using a clean disposable microtome blade for each block. The first and the last sections from each ribbon were subjected to light microscopic examination after routine hematoxylin and eosin staining.

The paraffin sections were transferred directly into the PCR tubes and incubated in 300 µL of xylene at 25°C for 5 minutes, pelleted at 12,000g for 5 minutes, resuspended in 300 µL of absolute alcohol at room temperature, spun down, and lyophilized. The pellets were then processed using the PUREGENE™ DNA isolation kit (Gentra Systems Inc, Minneapolis, MN, USA) according to

the manufacturer's instructions, which includes proteinase K (300 µg/mL) digestion overnight at 55°C. The final extracts were dissolved in TE buffer and kept at 4°C for later use.

PCR

The primers used to amplify the *Hsp65* gene (Figures 1 and 2), the *recA* gene and the *rpoB* gene are shown in Table 2. PCR was carried out in a DNA thermal cycler (GeneAmp PCR System 9600; Perkin-Elmer, Foster City, CA, USA). The reaction mixtures consisted of 0.5 µg of extracted DNA, 200 pmol of each of the oligonucleotide primers, 200 µM of each deoxynucleotide triphosphate (Boehringer Mannheim, Indianapolis, IN, USA), 1.5 mM MgCl₂, 1.25 U of *Taq* polymerase (Ampli*Taq* Gold™ DNA polymerase; Perkin-Elmer Cetus, Norwalk, CT, USA), 5 µL of 10× PE reaction buffer (500 mM KCl, 150 mM Tris-HCl [pH 8.0]), and high-pressure liquid chromatography-grade distilled water in a total volume of 50 µL. The reaction protocol, performed in a microcentrifuge tube, was as follows: (1) denaturation at 95°C for 10 minutes; (2) amplification for 45 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute; and (3) extension at 72°C for 10 minutes.

DNA sequencing and sequence analysis

The PCR products were sequenced using the ABI PRISM BigDye terminator cycle sequencing ready reaction kit and ABI PRISM 377 genetic analyzer (PE Applied Biosystems, Foster City, CA, USA). *Hsp65* sequences among various species of mycobacterium were retrieved from GenBank (<http://www.ncbi.nlm.nih.gov>). The accession numbers for these sequences are listed in Figure 2. Sequence analysis was performed using Vector NTI Suite 8 software (InforMax Inc, Frederick, MD, USA).

Results

Hsp65, first cloned from *M. tuberculosis* and sequenced in 1987,²⁴ is present in a wide range of

Table 2. Primers used in PCR to amplify the *Hsp65*, *recA* and *rpoB* genes

<i>Hsp65</i>	
Hsp65-A1	5'-ATG GTG TGT CCA TCG CCA AG-3'
Hsp65-B1	5'-CGC ATA CCC TCG GTG AGC TC-3'
<i>recA</i>	
recA-1	5'-AAG AGA CCC GCC AAC CGA TT-3'
recA-2	5'-CAT GTC GGC GAT CTC CAG GG-3'
<i>rpoB</i>	
rpoB-1	5'-GCG AGC TGA TCC AGA ACC AGA T-3'
rpoB-2	5'-ATC AGA CCG ATG TTC GGG CCT T-3'

mycobacterial species. To analyze the nucleotide polymorphism of *Hsp65* sequences, 87 strains of mycobacterium were retrieved from GenBank and aligned. The results showed a 424-bp segment containing a stretch of highly polymorphic sequences flanked by conserved regions, which are suitable for designing PCR primers (Figure 1). Furthermore, only 15 of 3828 ($87 \times 88/2$) comparisons could not be distinguished from each other by DNA sequence analysis (Figure 2). More specifically, these 15 indistinguishable 424-bp sequences could be divided into three identical groups: *M. farcinogenes* (AF547830) vs. *M. sene-*

galense (AF547872); *M. genavense* (AF547837) vs. *M. heckeshornense* (AF547843); and among *M. tuberculosis* (AF547885), *M. bovis* (AF547884, AF547813), *M. microti* (AF547856) and *M. africanum* (AF547803). Therefore, this analysis suggests that the 424-bp segment within the *Hsp65* sequence can be used to distinguish the majority of mycobacteria, supporting its qualification for the present study.

For each surgical specimen, DNA was separately extracted from both the granulomatous lesion and the normal tissue at the surgical margin, the latter of which served as a negative control.

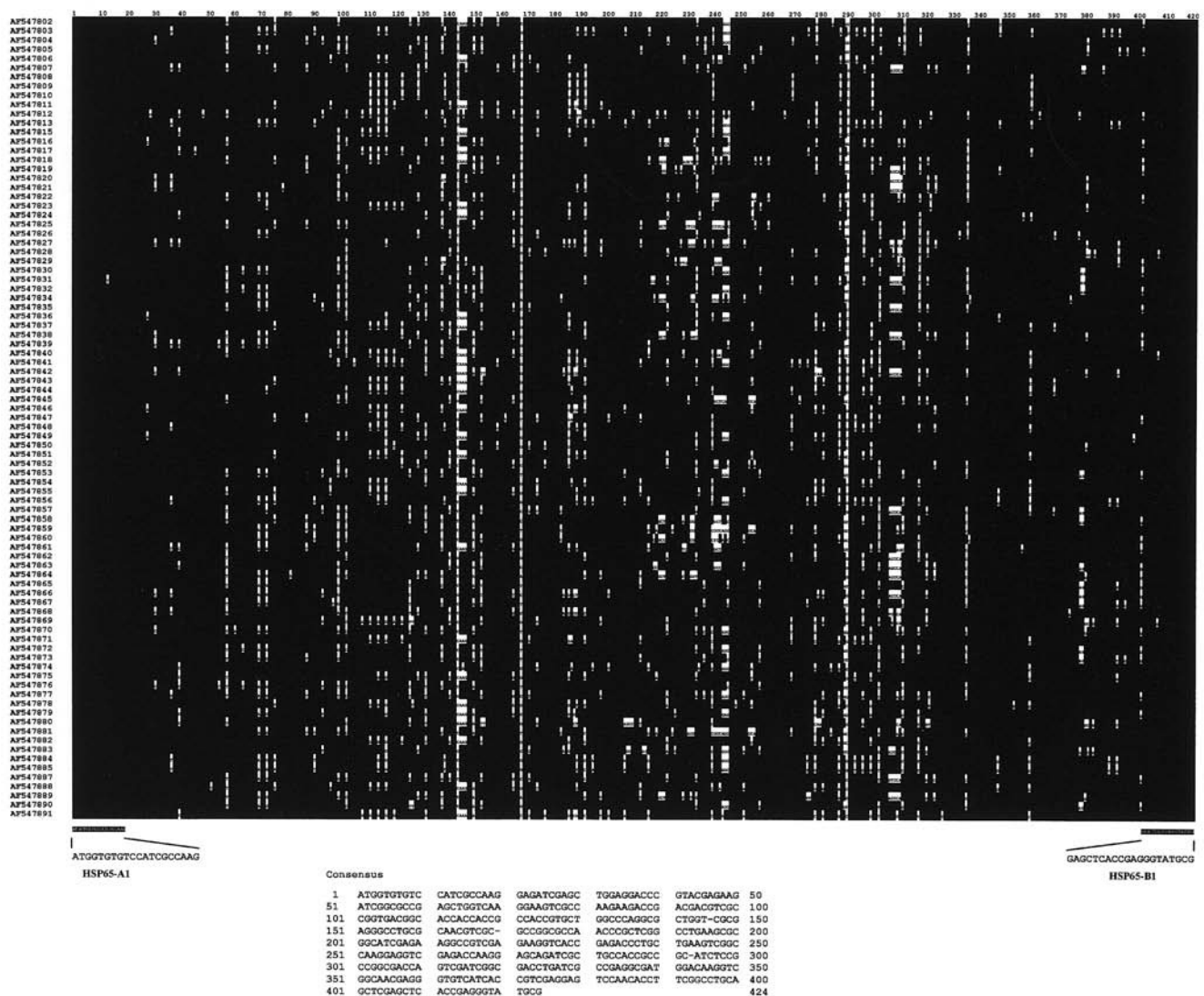


Figure 1. Comparison of the DNA sequences of the 65-kDa heat shock protein gene (*Hsp65*) of 87 mycobacterial strains (accession numbers shown on left) shows highly conserved flanking regions, which were used for PCR primers, designated Hsp65-A1 and Hsp65-B1. The consensus sequences, labeled as solid boxes in the field, are listed at the bottom of the figure.

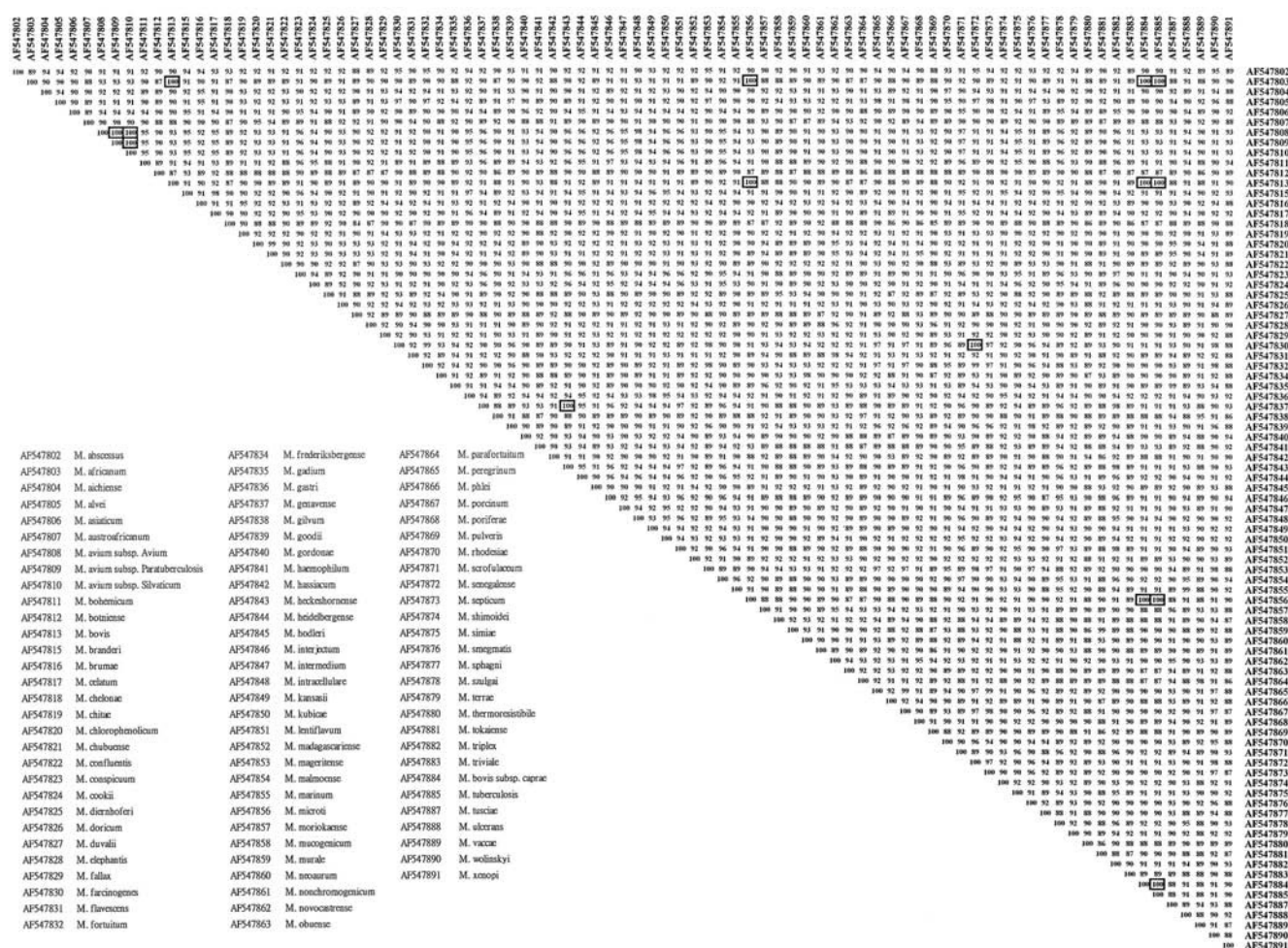


Figure 2. DNA sequence homology among 87 strains of mycobacterium. Each number represents the homology between two species/subspecies of mycobacterium, which are listed at the top and right side of the figure. Comparisons with 100% homology are boxed to highlight the identical *Hsp65* sequences between two species. (Mycobacterium species and corresponding access numbers listed in the left lower quadrant of the figure for cross-reference.)

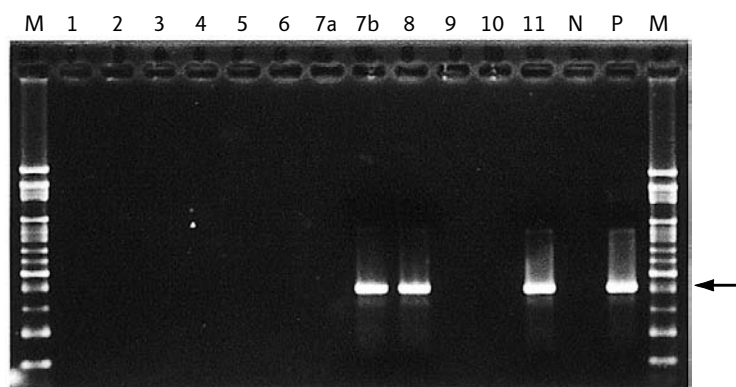


Figure 3. PCR-based detection of mycobacterial DNA from formalin-fixed, paraffin-embedded tissue. DNA templates extracted from the paraffin-embedded tissue were subjected to PCR for *Hsp65*. They yielded PCR products about 300 bp in size (arrow). The PCR products of each case are labeled on the top of the gel. Specimens #7a and #7b were collected from the same patient at different operations. M = molecular weight marker (100 bp); N = negative control; P = positive control from a known tuberculosis case.

Of the 12 specimens tested, three yielded PCR products specific to *Hsp65* that were visible under ultraviolet light on ethidium bromide stained agarose gel after electrophoresis (Figure 3). These PCR products were produced from DNA extracted from granulomatous lesions in specimens #7b, #8 and #11. In contrast, DNA extracted from the tissues at the surgical margin of each specimen yielded no PCR products (data not shown). Human β -globin gene could be amplified from all 24 DNA specimens, indicating successful extraction of DNA (data not shown).

All PCR products were sequenced and analyzed. Search of the GenBank database revealed that the amplicon sequences of specimens #7b and #8 were *M. tuberculosis* (99.7%) and that of

specimen #11 was *M. mucogenicum* (97.5% similar to AF547858; 98.6% to strains AF071135 and AJ307637) (Figure 4). To further substantiate the identified species from specimen #11, we examined two separate genes, *recA* and *rpoB*. The results showed that the 302-bp PCR product of *recA* was 98% similar to two known strains (AY286457 and AY286458) of *M. mucogenicum* (Figure 5A), and the 346-bp PCR product of *rpoB* was 99% similar to four known strains (AY147171, AY544946,

AY147170, AY147174) of *M. mucogenicum* (Figure 5B).

Discussion

The development of techniques for using formalin-fixed, paraffin-embedded specimens for PCR²⁵ has offered an opportunity to retrospectively examine if an occult mycobacterial infection is asso-

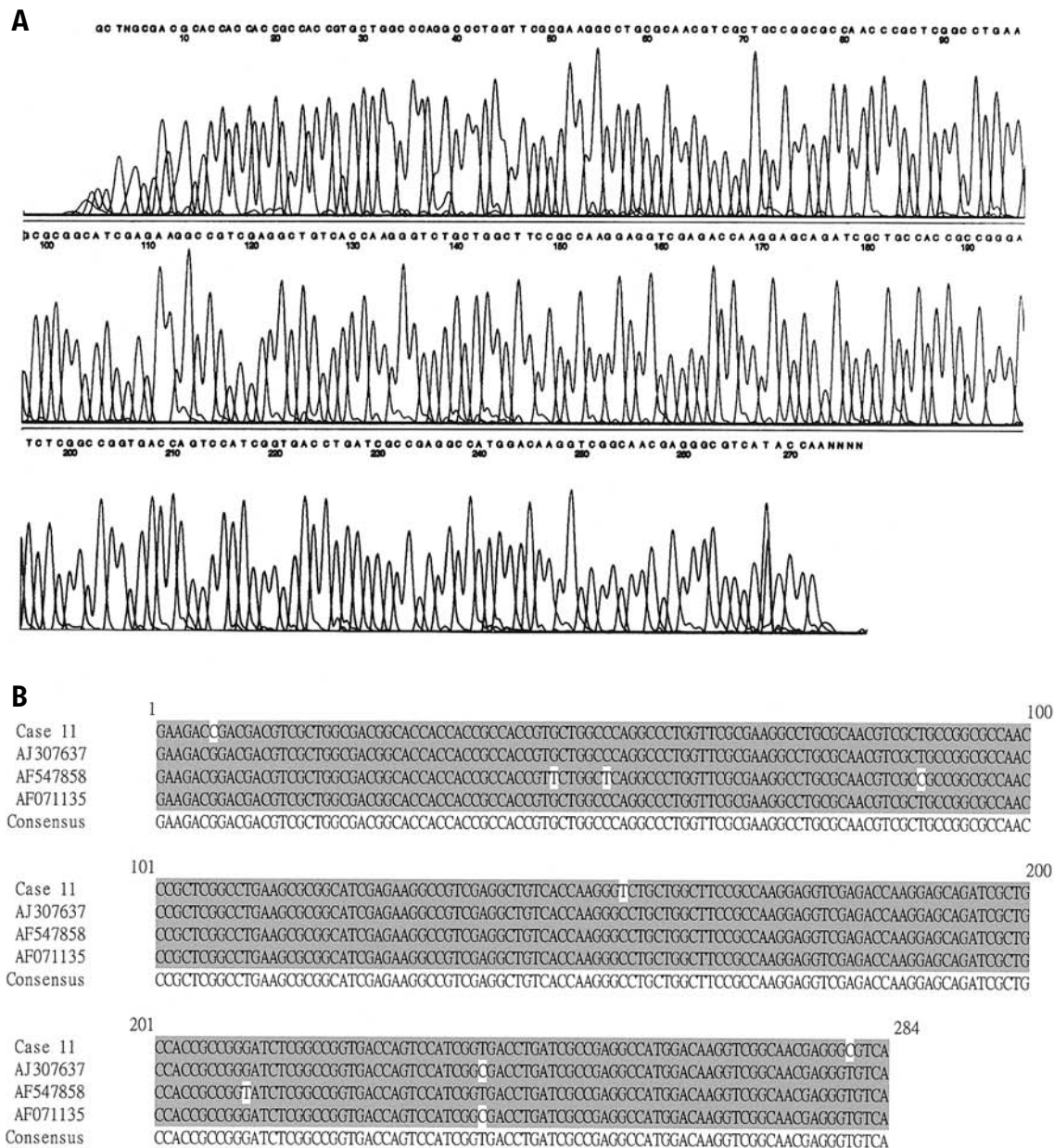


Figure 4. Case 11: (A) sequence chromatogram of *Hsp65* amplicon; (B) sequence alignment with three strains of *Mycobacterium mucogenicum* (AJ307637, AF547858, AF071135). The shaded nucleotides indicate consensus sequences.

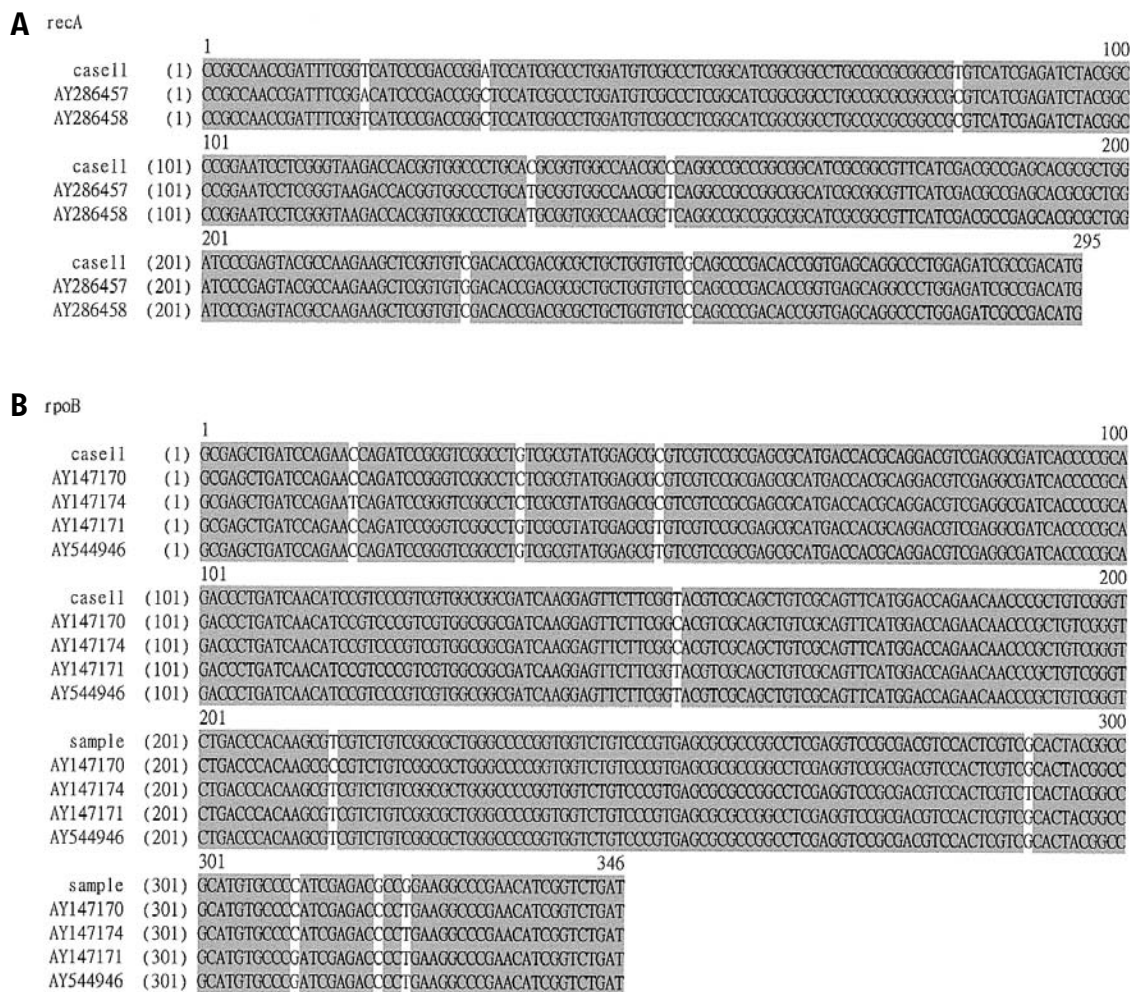


Figure 5. Alignment of (A) *recA* and (B) *rpoB* amplicons with the counterparts of known strains of *Mycobacterium mucogenicum*. The shaded nucleotides indicate consensus sequences.

ciated with an inflammatory disease. The present study has further demonstrated that identification of mycobacterium species is also possible by analyzing the DNA sequence polymorphism of *Hsp65*. Using this approach, we found that three of 11 cases of previously diagnosed Crohn's disease were associated with mycobacterial infection. The identified species was *M. mucogenicum* in one and *M. tuberculosis* in two, indicating that the designed *Hsp65*-based PCR can detect a broad spectrum of mycobacterium species in one reaction. Identification of the mycobacterium species involved in a given disease has important implications for treatment. The intrinsic problem of using such a broad spectrum primer pair is the associated low specificity, but this can be easily solved by DNA sequencing.

This is the first report of data showing an association between Crohn's disease and *M. mucogenicum*. This organism was designated as a new species in 1995 to replace the formerly named *M. chelonae*-like organism.²⁶ It belongs to the *M. fortuitum* complex and has been implicated in a number of nosocomial infections. It was initially isolated in 1982 from an outbreak of peritonitis,²⁷ and was later proved to be responsible for bacteremia^{28–30} and central venous line-associated sepsis.³¹ Recently, *M. mucogenicum* was shown to have caused granulomatous hepatitis in an autopsy case,³² indicating that it can induce granulomatous inflammation in the digestive system. In this study, tissues at the surgical margin of the resected bowel were used as controls. This would exclude the possibility of contamination, which

is a concern because *M. mucogenicum* has been a common contaminant in some laboratories.

We also identified two cases of tuberculosis (Cases 7 and 8), suggesting that ileocecal tuberculosis and Crohn's disease could be confusingly similar in terms of clinical and pathologic manifestations. Without the aid of modern PCR-based diagnosis, these cases would typically be diagnosed and treated as Crohn's disease due to the lack of caseous necrosis in the granulomas, negative acid-fast stain, and no growth of mycobacteria in culture. This result is in accordance with our previous findings that histopathologic variations of tuberculosis are fairly common and cause significant confusion or delay in diagnosis.²⁵ Therefore, for those cases with atypical morphology and negative stain/culture of acid-fast bacilli, PCR and DNA sequence analysis are the only available methods for distinguishing these diseases. A previous study showed that a PCR-based diagnostic method is capable of detecting 10 fg (equivalent to about 2 mycobacterial genomes) in a reaction,³³ and nine organisms of *M. tuberculosis* in a 5- μ m section of paraffin-embedded specimen.³⁴

Over a period of 12 years (1992–2003), 11 patients were diagnosed with and treated for Crohn's disease in this medical center, yielding a rough estimate of the annual incidence of Crohn's disease of about 0.78 per million population in Taiwan. This is considerably lower than that in the United States, where an annual incidence of 3 per 100,000 has been reported.³⁵ The disparity of incidences in various geographic regions could be due to a number of reasons, such as underdiagnosis of Crohn's disease in Taiwan or excluding those without a clearly identifiable etiology. If Crohn's disease is intended to be considered as a purely idiopathic disease of the bowel, the three cases with identifiable mycobacteria in this study should be excluded, which would yield an even lower estimated incidence of Crohn's disease in Taiwan. Since mycobacterial infection is a potentially curable disease, exclusion of those lesions associated with mycobacterial infection from Crohn's disease seems reasonable. The routine use of *Hsp65*-based PCR to exclude the possibility

of mycobacterial infection for every case with a diagnosis or suspicion of Crohn's disease is recommended.

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